REMARKS

Claims 1; 6; 13; and 38 to 42 remain in the application. Among these, claims 1 and 6 are independent method claims. Claims 7; 8; 10; 14; 15; 17; 18; 19; 20; 21; 22; 23; 25; 26; 27; 29; 31; 32; 34; 35; 36; and 37 were previously withdrawn as being directed to a non-elected invention or species. Applicant seeks the opportunity to consider reinstatement of certain withdrawn claims upon allowance of allowable subject matter.

Claims 1, 6, 13, and 38 to 42 were rejected under 35 U.S.C. § 112 ¶ 1 as failing to comply with the enablement requirement. Applicant maintains that the claims are enabled, as stated in the previous Office Action response of 12 January 2009. The Examiner has stated that the *in vitro* data of the present application is not sufficient to enable the current claims of the application. While the applicants disagree with the Examiner's assessment, the applicants have attached further data and evidence demonstrating *in vivo* the anti-cancer effects of the claimed invention. The data is attached in appendices 1 (methodology) and 2 (results) and is explained further, below.

In summary, xenograft experiments have been conducted in which nude mice have been implanted with cells from the human colon tumor cell line HT-29. The HT-29 cells form tumors which are then treated with the test compounds. Two perfluoralkyl compounds - closely related to the perfluorooctanoic acid of the claims - were investigated; PFBA (Perfluorobutyrate – C4) and PFHA (Perfluorohexanoic acid – C6). The HT-29 colon tumor cell line is fast growing in xenograft form and prone to ulceration.

The results show that both compounds have a beneficial effect on tumor rigidity. This is in keeping with the proposed mechanism of action of PFHA and PFBA as inducers of cellular differentiation, as discussed in the specification of the current application. Changes in tumor rigidity may become apparent through the generation of tumors with necrotic or fluid-filled centers and/or morphology changes in the cells within the tumor. In the case of PFHA, the experiment shows that a larger number of the tumors were fluid-filled compared to the tumors present in the vehicle treated control animals. In the clinical setting, changes in tumor rigidity result in shrinkage and/or degradation of the tumor tissue, possibly by cellular autophagy.

The data shows an *in vivo* effect of perfluoralkyl compounds on tumor rigidity and as such shows that perfluoralkyl compounds are useful in treating tumors *in vivo* and is consistent with the specification of the present application. As such, the attached information and data further confirm that the currently pending claims are indeed enabled.

It is also noted that the Examiner has made particular reference in the rejections that the IC50 values previously provided are not relevant as they do not include PFOA in table 2. The Examiner appears to be incorrect, as PFOA IC50s are clearly listed in table 2 (second listed compound) and therefore show the usefulness of the compounds in HT-29, MCF7 and PC3 cancer cell lines.

As such, the specification, along with currently submitted evidence and data, provide sufficient enablement for the current claims. That is, the present specification provides sufficient teachings that correspond to the claims. As is stated in the MPEP

> A specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as being in compliance with the enablement requirement of 35 U.S.C. 112, first paragraph, unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support....
>
> *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971). As stated by the court, "it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise, there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure." 439 F.2d at 224, 169 USPQ at 370.

MPEP § 2164.04

That is, while the Examiner has argued that various reports suggest that PFOA is a likely carcinogen and, therefore, it is not possible that the present claims are not enabled by the specification. Simply stating that an article may possibly come to a different conclusion does not mean that the claims are not enabled. Rather, the Examiner must state why it doubts that the evidence put forth by the applicants in the present application is truthful and accurate, and the Examiner must put forth evidence why the recited test results and data in the specification are not truthful and accurate.

In the same manner, the Examiner has suggested that because of the unpredictable nature of the prior art suggests, the provided data, examples, and testing procedures in the instant specification do not enable the claims. Once again, the Examiner must put forth evidence why the recited test results and data in the specification are not truthful and accurate and why the recited data is not predictive. That is, to substantiate the argument that the claims are not enabled, the

Examiner must put forth some evidence why someone following the testing procedures recited in the specification would get unpredictable results. The results in the specification are consistent with the results submitted with this amendment, and are consistent with what is being claimed. In fact, the predictability and repeatability of the disclosed data and experiments, as well as the verifiable beneficial results that were unexpected according to the prior art cited by the Examiner, further provides evidence that the presently claimed invention is novel over the prior art and should be afforded patent protection.

The applicants have provided sufficient data, procedures, and results that support the claims of the application. One having skill in the art would be able to follow the teachings of the application to arrive at the results cited in the specification. The supplemental data submitted along with this amendment further supports the fact that the specification enables the claims of the invention. Accordingly, the applicants request that the present claims be passed to allowance. Should the Examiner still maintain that the disclosed data is not sufficient to enable the claims, applicants request the Examiner particularly point out the inconsistencies of the presented data and the claims so that the applicants may provide further evidence to more directly address the Examiner's concerns.

Respectfully Submitted,

By_____
Patrick J. Fleis, Registration No. 55,185

RYAN KROMHOLZ & MANION, S.C.
Post Office Box 26618
Milwaukee, Wisconsin 53226
(262) 783 – 1300
2 September 2009
Customer No.: 26308
PJF:ar
Potter Clarkson\18803\090902 Amendment D.doc

Enclosures: Amendment Transmittal Letter
Return Postcard

3. MATERIALS AND METHODS

3.3 Animals

94 adult (5 – 9 weeks) female, athymic nude (*nu/nu)* mice will be obtained from Harlan, UK.

3.4 Animal Accommodation and Husbandry

Mice will be housed, up to 6 per cage, on sterile sawdust in sterile, solid-bottom, polypropylene cages.

The cages are individually vented units attached to a Techniplast Slimline Air Handling Unit. This unit maintains 70-80 air changes per cage, per hour, through HEPA air filters. Bedding will be changed once weekly in a laminar flow unit. The temperature will be maintained within a target range of 19-23° C and relative humidity of the IVC within a range of 40-70%. Twelve-hour periods of light will be cycled with twelve-hour periods of darkness.

3.5 Diet

Sterile RM1 diet (Special Diet Services Ltd., Stepfield, Witham, Essex, UK) will be used. The Medical School Resource Unit holds the specification of the diet. Deionised water will be autoclaved prior to use and changed at least once a week. Mice will be allowed water and diet *ad libitum* and will be acclimatised for at least 5 days prior to the study start.

3.6 Animal Health and Welfare

The mice have SPF-status and the housing and changing system assures that the SPF-status is preserved during the study. Trained personnel under supervision will handle the mice.

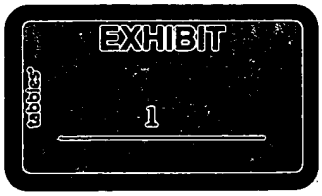
4. EXPERIMENTAL DESIGN

4.1 Identification of Animals and Allocation of *nu-nu* Mice into Experimental Groups

The animals will be randomly allocated to cages on arrival. Mice will be allocated into experimental groups (Table 1) once tumours have entered exponential growth phase (approximately 5mm in diameter; refer to section 4.4). The mice will be ear-numbered and weighed prior to the start of the experiment. The first mouse assigned to a cage will be individually identified by ear-numbering with the lowest number for that cage, the second mouse will be assigned the second (lowest) number and so on. An experimental card will be placed on each cage and will show the project licence code, test group, study number, sex and individual numbers of the mice within, and will identify the Home Office Licensee. In addition, these cards will be colour coded to correlate with the coding for the group.

4.2 Growth and Preparation of Cell Lines for Subcutaneous Injection into *nu-nu* Mice

Cell lines will be sub cultured according to the suppliers' instructions. Cultures will



be incubated in a humidified incubator at 37°C, 5% CO_2, until sufficient cells are available to implant the mice. Cells will be harvested, pooled, centrifuged, and re-suspended in cold medium. This will be mixed with an equal volume of cold Matrigel, so that the tumour cell injection solution will be a 50:50 mixture of tumour cells/medium and Matrigel for each cell line. Tumour cells will be injected in a volume of 100µl in a single flank only. The cell/Matrigel suspension will be aliquoted into pre-chilled Eppendorf tubes and kept on ice prior to injection. The period between preparation of the cell/Matrigel suspension and injection of tumour cells will not exceed two hours.

4.3 Implantation of Cell Lines

Cells will be harvested as detailed in section 4.2 and 100µl of cell suspension will be injected (s.c.) into a single flank of each mouse. The study will consist of 12 groups in total, each containing 6 - 10 mice per group.

1. Mice 47 – 52 will not be implanted with tumour cells but will be used for test dosing. (table 2)
    a. Mice will be administered 350mg/kg Perfluorobutyrate (mice 47 – 49; group 7) or 100mg/kg Perfluorohexanoic acid (mice 50 -52; group 8) daily by oral gavage for 4 days.
    b. Animals will be observed daily for signs of adverse drug reactions.
    c. On day 5, mice will be terminated and livers will be harvested and snap frozen for possible analysis.
2. Mice 53 – 94 will be implanted with HT-29 cells (table 2)
    a. HT-29 cells will be cultured using standard methods and implanted in a 100µL volume in a 50:50 mixture of cell suspension:Matrigel.
    b. Implantation density will be 1.75 x 10^6 cells per mouse (single flank; sc).
    c. Mice will be allocated into treatment groups once tumours reach approximately 4 mm in diameter.
    d. Mice 53 - 66 (group 9) will be administered vehicle (oral gavage daily for the duration of the study).
    e. Mice 67 – 80 (group 10) will be administered 350mg/kg Perfluorobutyrate (oral gavage daily for the duration of the study).
    f. Mice 81 – 94 (group 11) will be administered 100mg/kg Perfluorohexanoic acid (oral gavage daily for the duration of the study).
    g. Vehicle, Perfluorobutyrate, and Perfluorohexanoic acid will be administered in a dose volume of 10ml/kg bodyweight.
    h. Blood samples (approximately 10 microlitres, in duplicate) will be harvested from mice 53 – 94 once every fortnight to measure plasma concentrations of PFBA and PFHA.
    i. At termination, blood will be harvested by cardiac puncture (terminal plasma levels of PFBA/PFHA and clinical chemistry) and the liver and kidneys fixed in NBF. Tumours will be harvested, and pieces snap frozen in liquid nitrogen and also fixed in NBF.

Cell Line	Compound	Dose	Dosing Regimen	Group	Mouse Numbers
None	Perfluorobutyrate (PFBA)	350mg/kg	**Test Dosing:** Oral Gavage (daily for 4 days)	7	47 - 49
	Perfluorohexanoic acid (PFHA)	100mg/kg		8	50 – 52
HT-29	Vehicle	-	Oral Gavage (daily)	9	53 - 66
	Perfluorobutyrate	350mg/kg		10	67 - 80
	Perfluorohexanoic acid	100mg/kg		11	81 - 94

5. EXPERIMENTAL PROCEDURES

5.1 Bodyweight

The bodyweight of each mouse will be recorded upon allocation into groups and ear numbering, before implantation, once weekly following tumour implantation, and at the time tumours are measured.

5.2 Clinical Observations

Prior to the start of the study, all mice will be observed to ensure that they are physically normal and that they exhibit normal activity. Only normal mice will be allocated to the study. Following cell inoculation, each mouse will be observed twice weekly and a general assessment of condition recorded in the study diary. Animals will be terminated -refer to terminal procedures, section 5.5- if tumours become ulcerated or if the Home Office Project License moderate severity limit is exceeded.

5.3 Intercurrent Deaths

Any mouse requiring euthanasia during the study will be killed by an approved method (refer to terminal procedures, section 5.5). Information on intercurrent deaths will be entered into the study diary and will be reported to the Project Licence Holder as soon as possible.

5.4 Blood Sampling

Blood samples (approximately 10 microlitres, in duplicate) will be harvested from all mice once every fortnight to measure blood concentrations of administered compounds. Each 10 microlitre sample will be placed in an equal volume of water and immediately snap frozen pending analysis

5.5 Tumour Measurement

Tumour growth will be measured twice weekly for the duration of the study following cell implantation once tumours become palpable. Tumour diameters will be measured at four different sites - two lengths and two widths -using a digital slide gauge. This will be increased to three lengths and three widths if the tumour is an irregular shape.

Tumour volumes will be calculated using the formula: $4/3\ \pi\left(\frac{d1 + d2}{4}\right)^3$

Where d = mean diameter (n = 2)

Animals will be terminated if tumour volumes exceed 1.44cm^3 or if they become ulcerated.

5.6 Terminal Procedures

On the day of termination tumour volumes will be recorded and the mice will be weighed and transferred to the post mortem room. The animals will be killed by exposure to a rising concentration of CO_2 and cervical dislocation.

5.6.1 Mice 1 – 44 and 52 - 87

Blood will be harvested by cardiac puncture and transferred to heparinised tubes for plasma preparation. Tumours, livers and kidneys will be processed as follows:

Tumours

- Each tumour will be removed and the weights recorded.
- The tumour will be cut in half and one half placed in 10 % neutral buffered formalin (NBF) then prepared for paraffin embedding (section 6.4) for histochemical analysis
- The remaining half, or a large representative sample, will be placed in a cryovial and flash frozen.

Liver

- The liver will be removed and the weight recorded.
- 2 slices of liver (approximately 2 mm strips) will be taken, one from the Left lobe and one from the Median lobe. These will be placed in 10 % neutral buffered formalin (NBF) then prepared for paraffin embedding (section 6.4) for possible future histochemical analysis.

Kidneys

- The kidneys will be removed and the weights recorded; kidneys will be weighed together.
- The right kidney will be cut in transverse section and the other will be cut longitudinally.
- Half of each kidney will be taken and placed in 10 % neutral buffered formalin (NBF) then prepared for paraffin embedding (section 6.4) for possible future histochemical analysis.

5.6.2 Mice 45 – 51 and Mice 88 - 94

Tumours will be harvested and snap frozen for protein and RNA extractions as detailed below.

- Two pieces of tumour (size as detailed in the MSRU) will be immediately flash frozen in liquid nitrogen in a cryovial, and then stored at approximately -70 °C for protein preparation.
- Two pieces of tumour (size as detailed in the MSRU) will be immediately flash frozen in liquid nitrogen, using a separate cryovial for each piece, then stored at approximately -70 °C for RNA extraction.
- Remaining tumour tissue, or a large representative sample, will be placed in a cryovial and snap frozen for possible future analysis.

6. Laboratory Procedures

6.1 Plasma Preparation

Following removal into tubes suitable for plasma preparation, venous blood samples will be mixed on a roller for 10 minutes. Red blood cells will be removed by centrifugation at 2,000 – 3000 rpm for 10 minutes at 8 – 10 °C. The supernatant (plasma) will be transferred to a second tube and stored at approximately -70 °C until required for analysis.

6.2 Clinical Chemistry

Plasma will be analyzed using a Cobas Integra 400 analyzer. Kits purchased from Roche Diagnostics GmbH will be used to measure ALT (cat # 20764957322), AST (cat # 20764949322), BUN (cat # 20763039322) and creatinine (cat # 03263991190).

6.3 Plasma Levels of Administered Compounds

Blood- (in-life) and plasma- (terminal) levels of administered compounds will be measured using appropriate methodology (LC/MS/MS).

6.4 Preparation of tissues for paraffin embedding

Liver and kidney samples will be placed immediately into NBF for 36-45 hours. Tissues will be processed according to CXR method Pat-005. The tissues will be embedded in paraffin wax according to CXR method Pat-006 for possible future histochemical analysis (H&E staining and immunohistochemisry; details will be discussed in the study report, as appropriate).

CONTENTS

List of Tables

List of Figures

Appendices

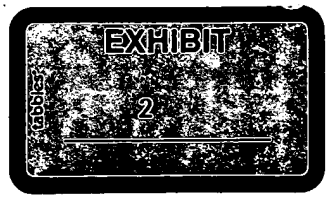


Table 1: CXR0788 HT-29 Xenograft Study Pre-Treatment Details	
Cell Line	HT-29
Mouse Details	Female, ICRF-Foxn1nu, 5 – 9 weeks, Harlan.
Implantation Density	1.75×10^6
Number of mice implanted with tumour cells	42
No-takes	0
Tumours that failed to enter exponential growth	0
Tumour excluded based on excessive size	5
Ulceration	0
Mice found dead prior to treatment	0
Tumours in inaccessible position	0
Number of days taken for tumours to enter exponential growth phase	19
Number of Tumours / mice allocated into study groups	37 (13 x vehicle; 12 x PFBA; 12 x PFHA)

NB. Test dosing in tumour-free nude mice was performed prior to the initiation of dosing. Refer to Appendix 1 for details of the dosing regimen and outcome.

Table 2: Efficacy of PFBA and PFHA in HT-29 Xenografts	Vehicle	250mg/kg PFBA	100mg/kg PFHA
Day treatment initiated	22	22	22
Mean tumour size at start of treatment ± SEM	215.04 ± 21.604 (n = 13) (range = 124.79 – 318.72)	212.089 ± 28.634 (n = 12) (range = 58.82 – 369.14)	175.956 ± 19.948 (n = 12) (range = 85.94 – 310.36)
Number of Tumours/Mice	13	12	12
Dosing Regimen	Daily oral gavage	Daily oral gavage	Daily oral gavage
Treatment Phase	Days 22 – 63 (42 doses). Terminated day 64.	Days 22 – 63 (5 doses at 350mg/kg and 36 doses at 250mg/kg; no dose on day 7). Terminated day 64	Days 22 – 63 (41 doses). Terminated day 64.
Slow growth rate (but completed study)	3	2	2
Tumour regression	1	0	1
Tumour Ulceration (mice lost mid-study)	3	4	4
Excessive Tumour Size (mice lost mid-study)	5	4	4
Loss of mice (injury; treatment -related etc)	0	1 (found dead; probably treatment related). This mouse was found dead following 5 doses at 350mg/kg. Mice in this group were allowed to recover for 24hrs then dosing resumed at 250mg/kg for remainder of study.	0
Mean tumour volume at final tumour measurement on day 61. ***(NB, no tumour volumes recorded on day 64)*** **(mm^3 ± SEM; range)**	549.813 ± 127.144 (n = 5) (range = 172.02 – 854.88)	562.865 ± 133.169 (n = 3) (range = 356.84 – 812.05)	850.328 ± 327.36 (n = 6) (range = 159.26 – 2065.34)
Tumour Weight (g ± SEM; range)	1.008 ± 0.156 (n = 11) (range = 0.25 – 2.042)	0.850 ± 0.158 (n = 11) (range = 0.294 – 1.828)	1.105 ± 0.225 (n = 11) (range = 0.205 – 2.594)
In-life levels of Test Item (µM ± SD)	NOT TESTED	**5 Doses**: 67.12 ± 42.98 **20 Doses**: 128.05 ± 42.81 **34 Doses**: 150.67 ± 41.90	**7 Doses**: 3.89 ± 2.45 **21 Doses**: 4.73 ± 4.10 **35 Doses**: 5.52 ± 5.91
Terminal Plasma Levels of Test Item (µM ± SD)	NOT TESTED	**9 - 40 Doses**: 197.71 ± 60.89	**10 - 41 Doses**: 1.64 ± 0.65
Tumour Levels of Test Item (µM ± SD)	NOT TESTED	**9 - 40 Doses**: 57.97 ± 12.13	**10 - 41 Doses**: 6.06 ± 7.40
Number of mice/tumours that completed the study up to day 64 (41 or 42 doses of compound; includes those with slow growth and those that regressed)	5	3	5

Treatment related effects: **Tumour growth and structure/morphology**	-	Due to loss of data points (tumour ulceration etc) the effect of Test Compound on tumour volume was unclear. Tumours were noted to be softer than controls and the tumour rigidity scale was significantly higher in these treated mice.	Due to loss of data points (tumour ulceration etc) the effect of Test Compound on tumour volume was unclear. Tumours were noted to be softer than controls and PFBA-treated tumours and the tumour rigidity scale was significantly higher in these treated mice compared to the other two treatment groups.
Treatment related effects: Bodyweight	-	No clear effect	No clear effect
Treatment related effects: Clinical Chemistry	-	No significant increase in ALT, AST, BUN or creatinine. Plasma creatinine significantly lower than controls	No significant increase in ALT, AST, BUN or creatinine. Plasma AST significantly lower than controls
Treatment related effects: Organ Weights	-	Increased liver weights Increased kidney weights	Increased liver weights
Treatment related effects: Histopathology. ***NB Expert histopathological analysis was not performed on liver, kidney or tumour sections; any compound related changes noted were obvious to the untrained eye.***	-	Adverse histopathology in the liver. No obvious adverse histopathology in kidneys	Adverse histopathology in the liver No obvious adverse histopathology in kidneys 3 out of the 12 tumours had large holes in their centres' and 2 out of the 12 had smaller holes evident within the sections.

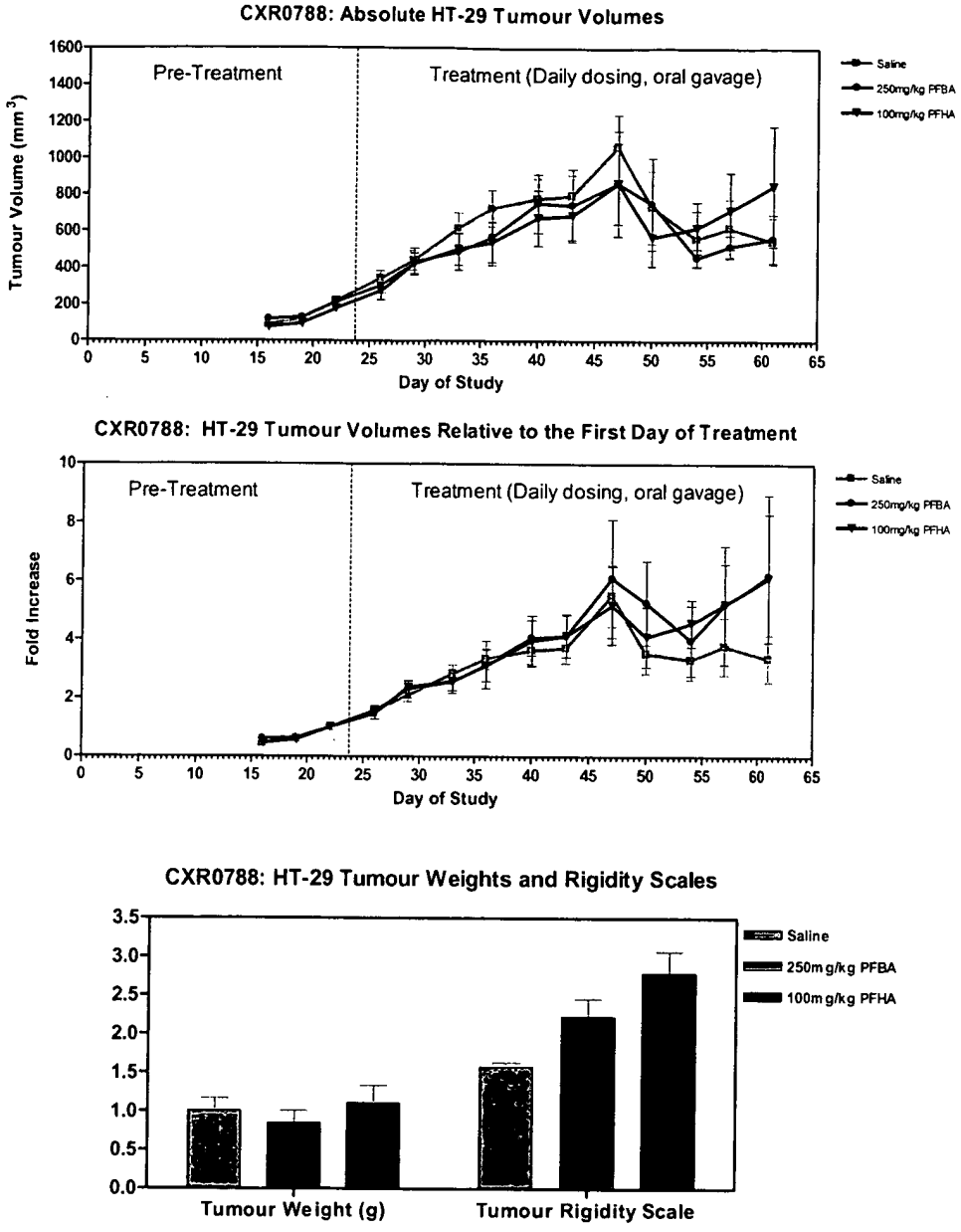
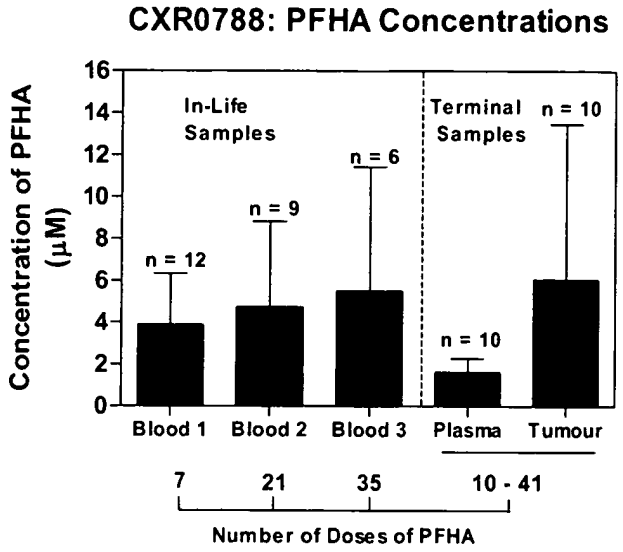
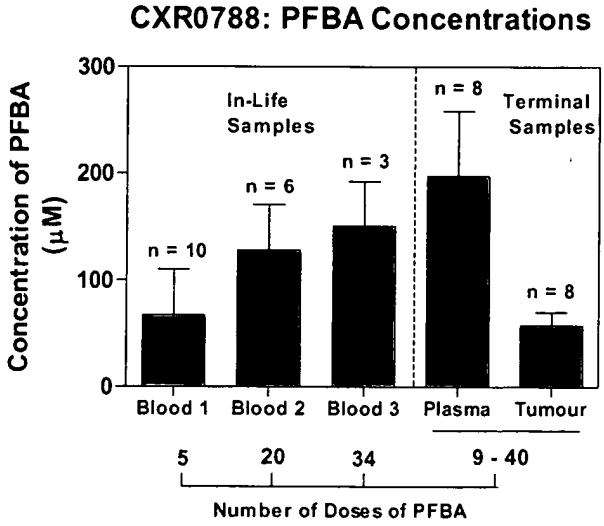


Figure 1: HT-29 Tumour Volumes (absolute and relative), tumour weights and rigidity scales.

The loss of data points (tumour ulceration etc) throughout the study made the in-life tumour volume data difficult to interpret; the effect of Test Compound on tumour volume therefore remains unclear. Tumour rigidity measurements indicated that there may have been compound-related effects on tumour structure: the highest TRS values (and the greatest degree of 'give') were seen in PFHA treated tumours.



Terminal plasma and tumour levels of PFHA values were considered outliers and were excluded from calculations of Mean Test Compound concentrations (mice 87 and 90).



Terminal plasma and tumour levels of PFBA were very high in two mice (68 and 76) and very low in one mouse (82). These values were considered outliers and were excluded from calculations of Mean Test Compound concentrations.

Figure 2: Test Item levels in mouse and tumour tissues.

Exposure to PFBA reached moderate to high levels for the duration of the experiment. Exposure to PFHA was significantly lower; this may reflect the lower dose (100mg/kg compared to 250mg/kg) and differences in the clearance rate and half life of this compound.

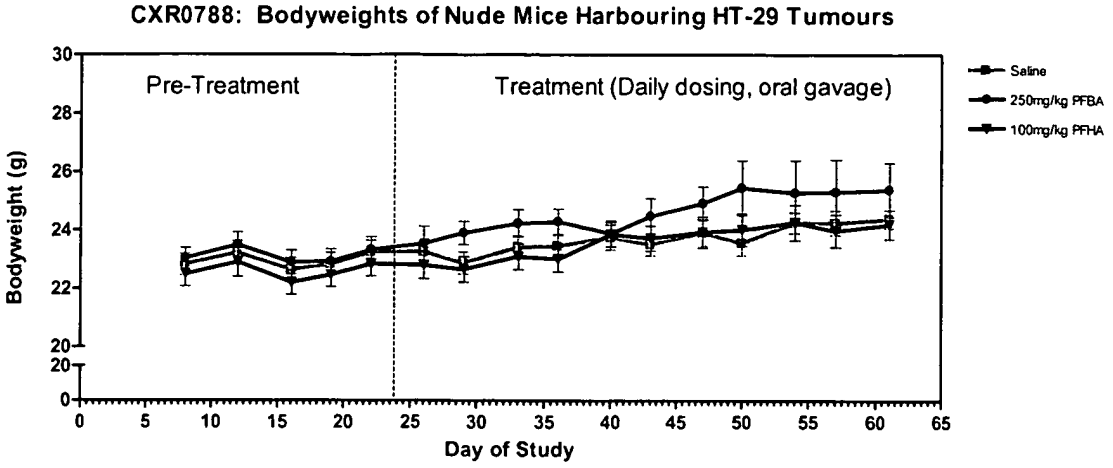


Figure 3: Bodyweights of nude mice administered saline or Test Item.

There was no effect of test compound on mean mouse bodyweights. Again, it should be noted that there was continual loss of data points over the study period that may have masked any compound-related effects.

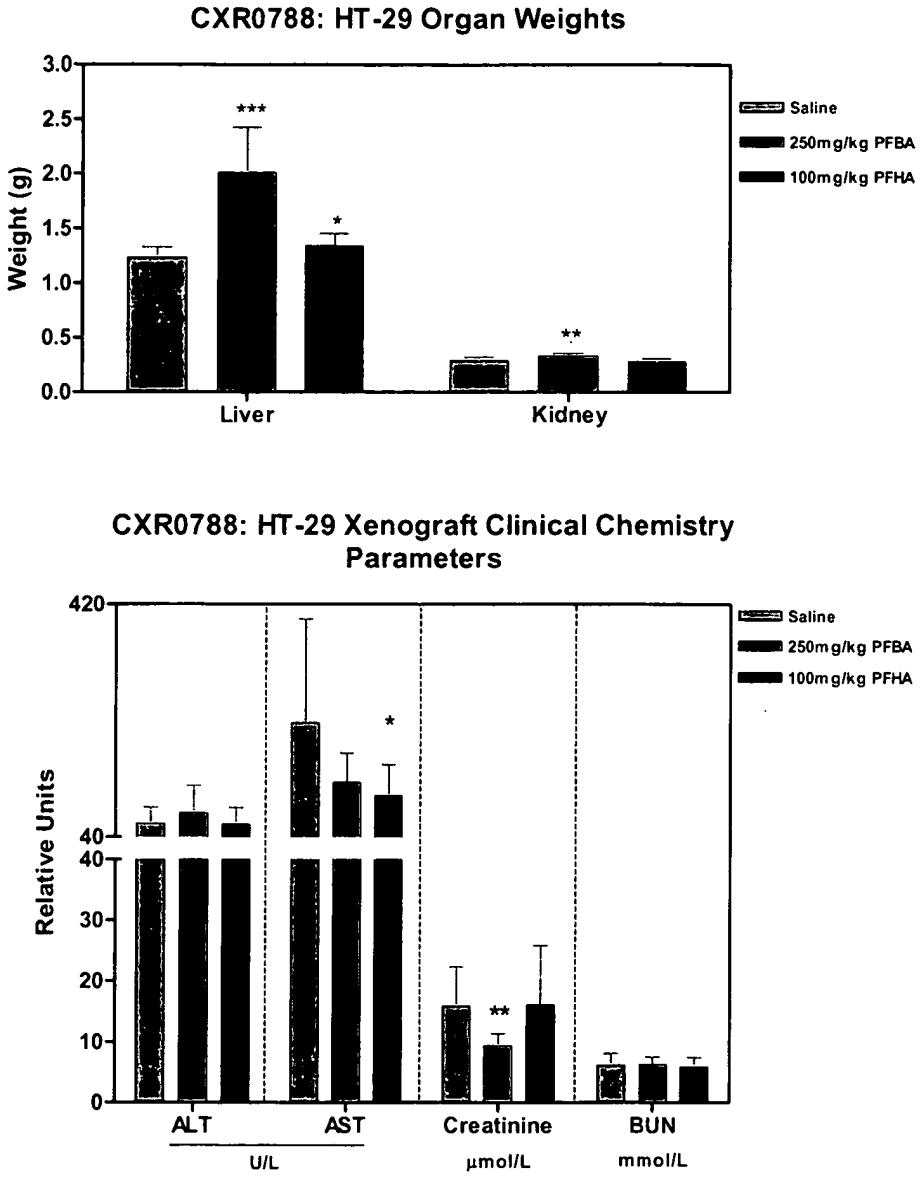


Figure 4: Nude mouse organ weights and plasma markers of liver toxicity (ALT and AST) and kidney toxicity (creatinine and BUN).

Liver weights were significantly higher in mice administered PFBA and PFHA compared to controls. There was, however, no corresponding increase in plasma ALT or AST. H&E staining (see figure 5) indicated that there was some liver damage, so it is possible that the time point selected to measure ALT and AST (approximately 16 hours post dosing) may not have been optimal to measure these markers of liver damage. There was a slight reduction in plasma creatinine in mice administered PFBA, but the significance of this remains unclear – no obvious signs of kidney damage were evident following H&E staining.

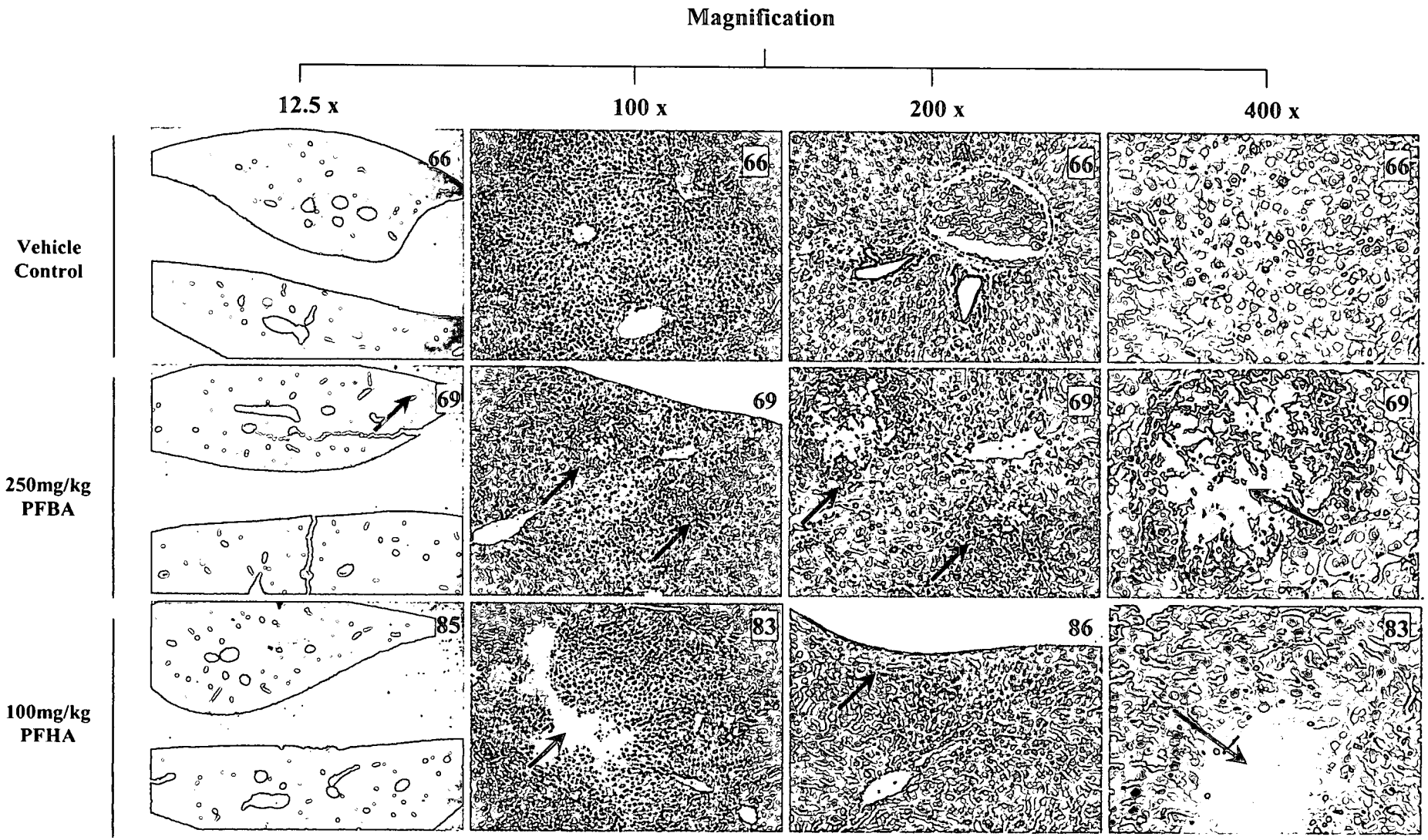


Figure 5: H&E staining of liver sections photographed at various magnifications.

Mouse numbers are highlighted in grey. Mouse 66 (control), mouse 69 (PFBA) and mice 83, 85 and 86 (PFHA) samples were selected as representative sections for each treatment. Adverse histopathology was noted in the livers of PFBA and PFHA mice (arrows). It should be noted that there were no corresponding increases in mean transaminase levels in the plasma of treated mice.

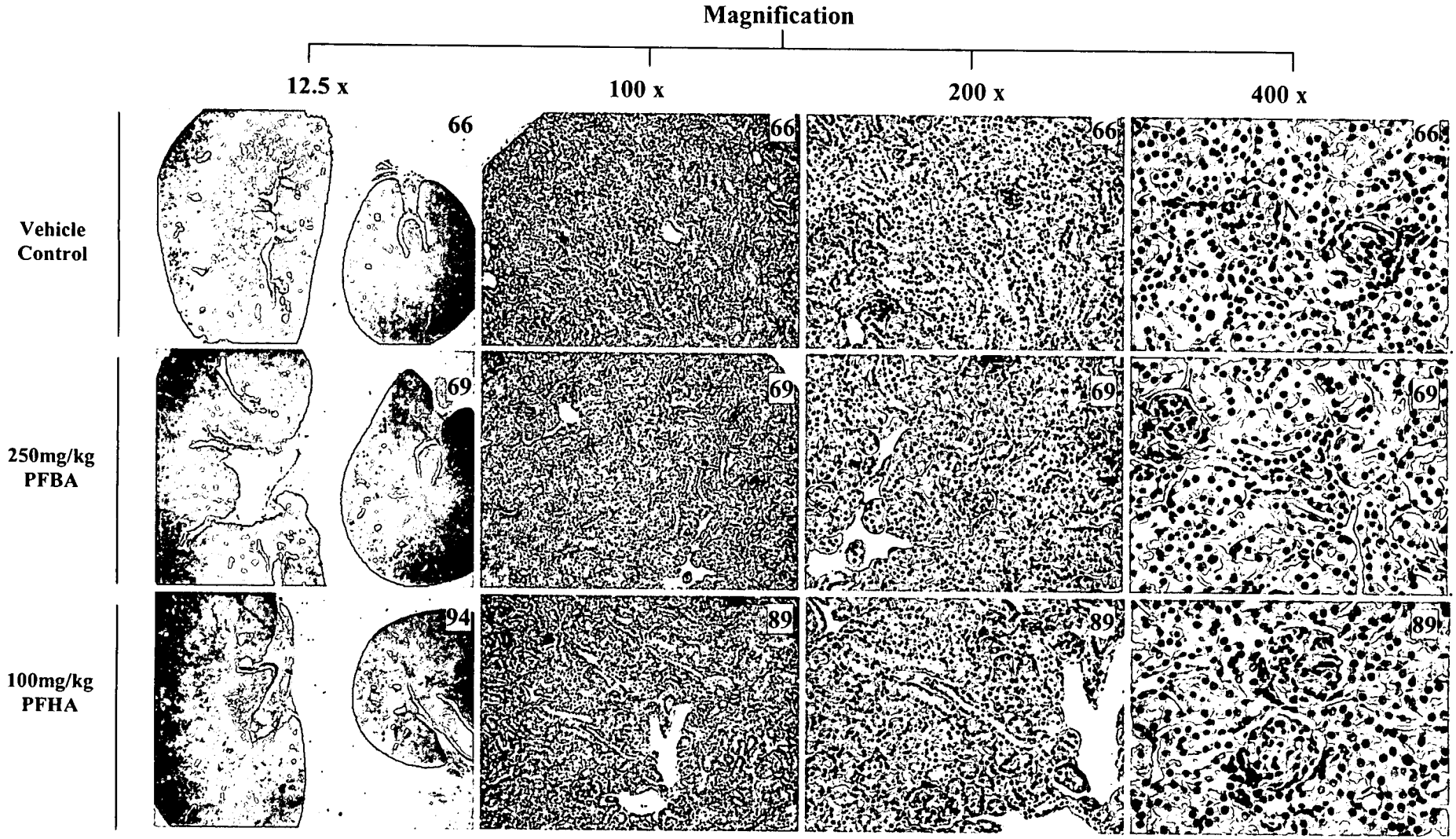
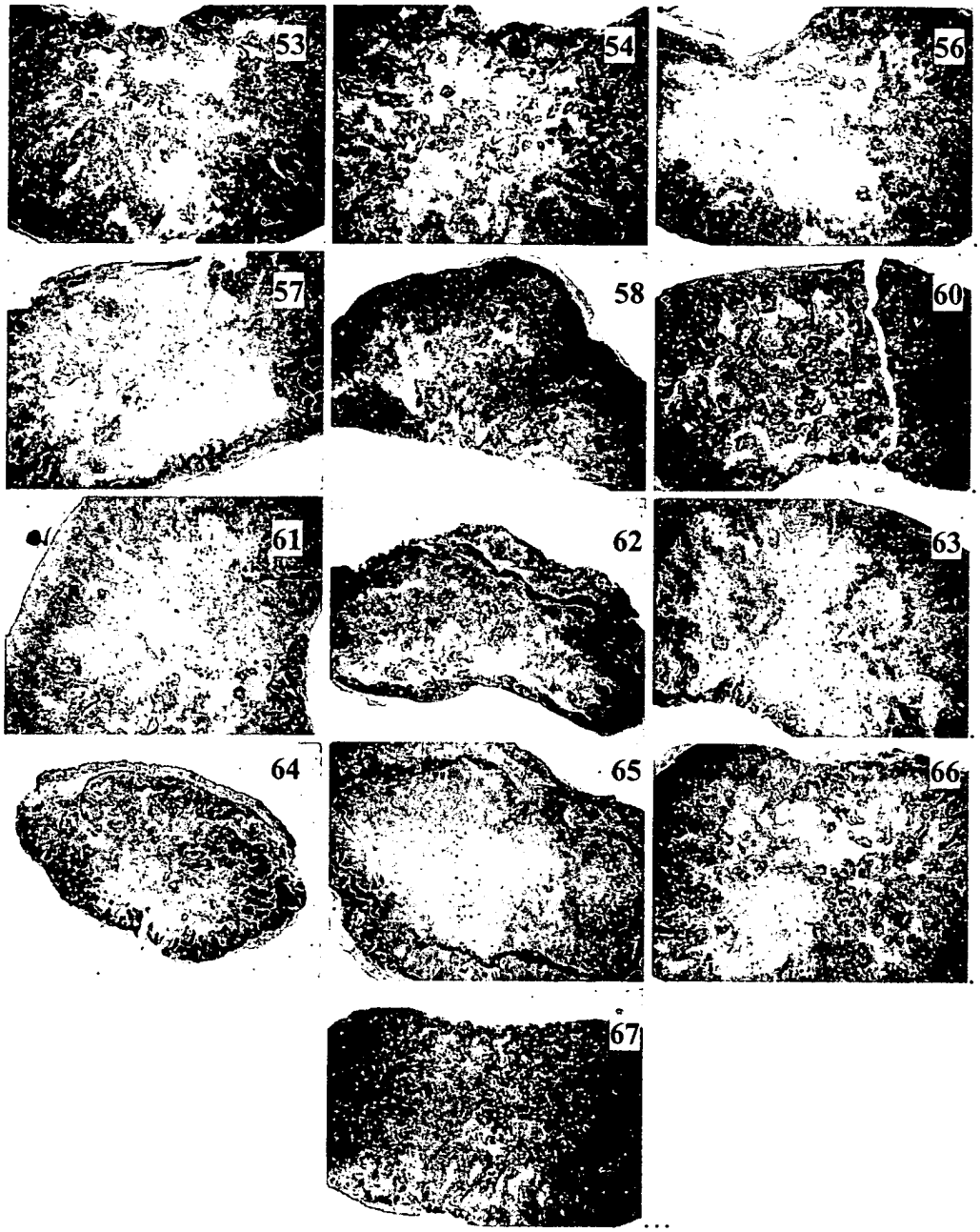


Figure 6: H&E staining of kidney sections photographed at various magnifications.

Mouse numbers are highlighted in grey. Mouse 66 (control), mouse 69 (PFBA) and mice 85 and 94 (PFHA) samples were selected as representativ sections for each treatment. No obvious adverse histopathology was noted in the kidneys.

Saline Control Tumours at Low Magnification (x 12.5)



Saline Controls at Higher Magnification (x 100)

Figure 7: H&E Staining of control tumour sections at low and high magnification

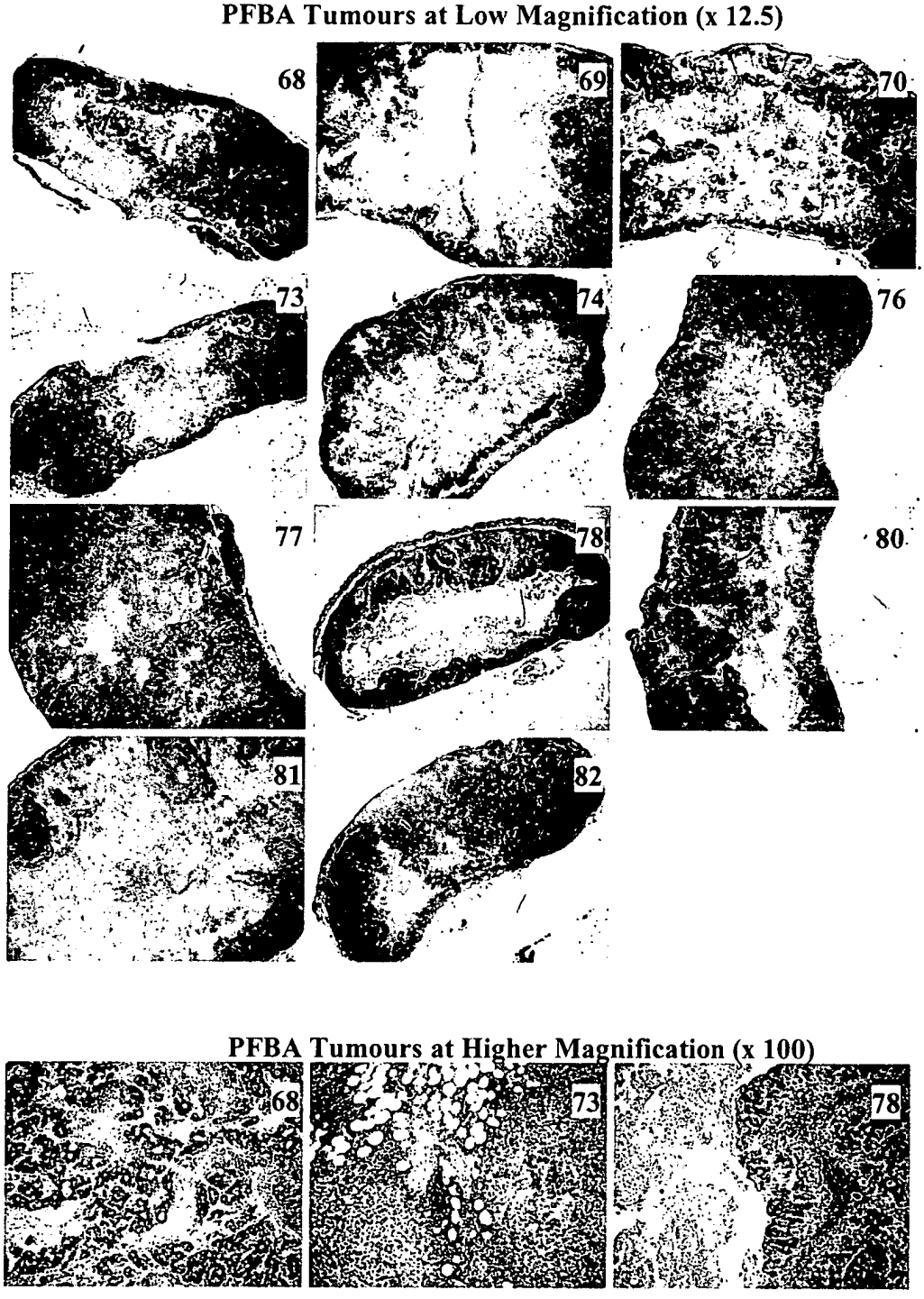


Figure 8: H&E Staining of PFBA tumour sections

There were holes evident in some tumour sections.

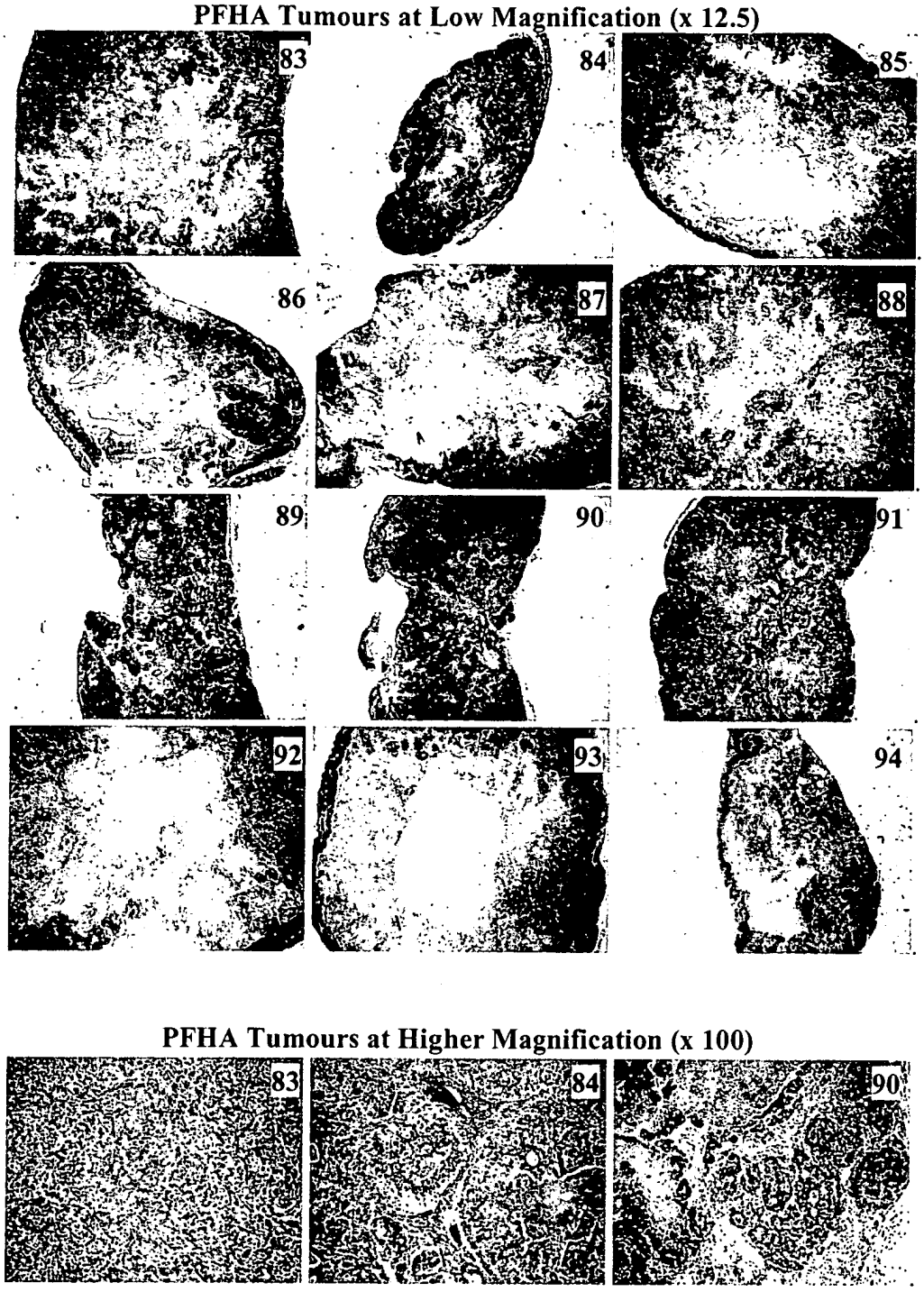


Figure 9: H&E Staining of PFHA tumour sections

There were holes evident in some tumour sections.

APPENDIX 1: Test Dosing Details

Treatment	Mouse ID	Daily Bodyweights (g) over 4-day test dosing period (days 12 - 15 of study)				In-Life Observations	Observations at Necrposy
		12	13	14	15		
350mg/kg PFBA	47	23.3	23.4	23.4	21.8	Mouse dosed on days 12, 13 and 14. No dose on day 15. Animal was lethargic and cold and o/n bodyweight loss was noted. Mouse euthanized on day 14.	Intestines distended by gas up to 10mm in diameter. Liver: wt 1.281g, pale, focus on median lobe (8 x 4mm). No ruptures. No lung dose. Both kidneys pale.
	48	23.7	24	24.2	24.2	NAD. Dosed and terminated as per protocol on day 15	Liver and kidneys appeared normal. Stomach almost empty. Proximal half of SI empty; distal SI contents normal. Caecum and contents normal. Large intestine normal.
	49	25.1	24.1	25	25.4	NAD. Dosed and terminated as per protocol on day 15	Liver and kidneys appeared normal. Stomach almost empty. Proximal half of SI empty; distal SI contents normal. Caecum and contents normal. Large intestine normal.
100mg/kg PFHA	50	23.7	24.1	23.9	23.9	NAD. Dosed and terminated as per protocol on day 15	Liver and kidneys appeared normal. Stomach almost empty. Proximal half of SI empty; distal SI contents normal. Caecum and contents normal. Large intestine normal.
	51	25.8	25.4	24.9	25.8	NAD. Dosed and terminated as per protocol on day 15	Liver and kidneys appeared normal. Stomach almost empty. Proximal half of SI empty; distal SI contents normal. Caecum and contents normal. Large intestine normal.
	52	26.8	26.9	26.5	26.4	NAD. Dosed and terminated as per protocol on day 15	Liver and kidneys appeared normal. Stomach almost empty. Proximal half of SI empty; distal SI contents normal. Caecum and contents normal. Large intestine normal.
	95	21.5	21.5	21.5	21.6	NAD. Dosed and terminated as per protocol on day 15	Liver and kidneys normal. SI findings as for 50, 51 and 52, but some gaseous build-up either side of the caecum (4mm in diameter at its biggest).

Tumour-free nude mice were administered Test Compounds by oral gavage (saline vehicle) for 4 consecutive days and terminated the next day, unless otherwise indicated in the table. NAD = no abnormalities detected; SI = small intestine.

APPENDIX 2: Tumour Rigidity Scale

1. Tumour firm to the touch. Obvious nodules resistant to firm pressure.
2. Slight loss of rigidity. Change may be noticeable to the animal handler during tumour measurement.
3. Soft: yields to moderate pressure.
4. Yields to fairly gentle pressure. Obvious indents seen when calipers applied during tumour measurement.
5. Bursts at measurement or dissection.

APPENDIX 3: Individual Control Mouse Data Points

HT-29 Tumours							Tissue Concentrations of Test Compound (µM)					Clinical Chemistry Parameters				Terminal Measurements (g)			Tumour Details (NBF-Fixed)		
Treatment	Mouse ID	Study Diary Comments/SSO and SO Comments	Date Mouse Terminated	Day of study	Reason for Termination of mouse	Number of Doses of Saline	Blood Harvest 1: Day 29	Blood harvest 2: Day 43	Blood harvest 3 Day 57	Terminal Plasma: Day 64	Tumour: Day 64	ALT (U/L)	AST (U/L)	CRE (µmol/L)	BUN (mmol/L)	Body wt	Liver wt	Kidney wt	Tumour wt	TRS	Morphology
Saline	53		11/05/2009	47	Excessive tumour size	25	Not tested					93.8	252.6	14.2	8.78	23.7	1.083	0.243	1.307	1.5	Approximately 50% of the tumour exterior is covered by skin. Small amount of scab visible on the uppermost surface. Tumour pale in colour with some white areas visible on the interior of tumour. Possible evidence of blood vessels on the exterior surface.
	54		11/05/2009	47	Excessive tumour size	25						93.6	252.4	16	8.82	27	1.302	0.322	2.042	1.5	Approximately 50% of the tumour exterior is covered by skin. Large scab visible on the uppermost surface. Tumour pale in colour with some white areas visible on the interior of tumour. Possible evidence of blood vessels on the exterior surface.
	56		11/05/2009	47	Ulcerated tumour	25						73.6	308	28.8	9.4	24	1.172	0.294	1.035	1.5	Entire exterior surface of the tumour is covered by skin. Large scab visible on the uppermost surface. Tumour pale in colour with possible evidence of blood vessels on the underside of tumour. The vast majority of the cross section is composed of white material
	57	At termination, bled into thoracic cavity. Sample not taken directly from heart and may have clotted.	28/05/2009	64	Completed study	42						84.8	603.4	15.4	4.18	23.9	1.166	0.258	0.878	1.5	Approximately 50% of the tumour exterior is covered by skin. Scab visible on the uppermost surface. Possible evidence of blood vessels on the underside of the tumour. The tumour is pale in colour with the majority of the cross section whiter than the surrounding tissue.
	58		28/05/2009	64	Completed study	42						30.2	62.1	9.9	5.19	24.7	1.308	0.288	0.581	1.5	Small patch of skin on the tumour exterior. Possible evidence of blood vessels on the exterior of tumour. Tumour is pale in colour with the majority of the cross section paler than the surrounding tissue.
	60	Liver, kidney and tumors harvested but weights not recorded (NR) in error	26/04/2009	32	Ulcerated tumour	10						38	100.2	15.8	4.12	23.1	NR	NR	NR	1.5	Majority of the exterior surface is covered in skin . Evidence of scab on the uppermost surface. Possible evidence of blood vessels on the exterior surface. Possible damage to interior by scab. Some patches of white material visible on cross section.
	61	Mouse had second smaller tumour that was first noted on day 31. (*Study director's note: H&E analysis indicated this was lymph tissue rather than a small tumour.*)	14/05/2009	50	Excessive tumour size	28						91.8	140	14.2	5.36	28.7	1.208	0.268	1.449 (tumour) and 0.279 (lymph)	1.5	Tumour were present approximately 50% of the tumour exterior is covered in skin with some possible evidence of blood vessels on the exterior of the tumour. The tumour is pale in colour, most of the cross section is whiter than the surrounding tissue. Lymph tissue: possible evidence of blood vessels on the exterior surface, the cross section shows small amount of white material on the interior.
	62	Pale focus in middle lobe of liver noted at necropsy.	28/05/2009	64	Completed study	42						28.2	82.2	10.8	6.34	25.6	1.367	0.299	0.25	1.5	A large scab was present on the exterior surface of the tumour accompanied by a small amount of skin. Darker scab material is present on the interior of the tumour. The rest of the tumour is pale in colour. Approximately 50% of the cross section shows whiter material on the interior.
	63		11/05/2009	47	Excessive tumour size	25						83.7	251.8	16.1	8.19	24.9	1.176	0.271	1.177	1.5	Approximately 50% of the tumour exterior is covered by skin with possible evidence of blood vessels on the exterior of the tumour. The tumour is pale in colour with the majority of the cross section showing whiter material on the interior.
	64		28/05/2009	64	Completed study	42						36	106.4	16	4.48	25	1.395	0.342	0.308	2	Approximately 30% of the tumour exterior is covered with skin. The tumour is pale in colour and the cross section shows a white core at the centre.
	65		28/05/2009	64	Completed study	42						33	86.4	9.4	5.7	23.5	1.232	0.327	0.938	2	Approximately 30% of the tumour exterior is covered by skin. Scab damage is visible on the uppermost surface, with some protrusion into the interior. Possible evidence of blood vessels on the exterior of the tumour. The tumour is pale in colour with the interior whiter in colour than the surrounding tissue
	66		11/05/2009	47	Excessive tumour size	25						66	250.4	29.6	4.28	22.5	1.152	0.268	1.127	1.5	Approximately 50% of the tumour exterior is covered by skin. Scab damage is visible on the uppermost surface, with some protrusion into the interior. Possible evidence of blood vessels on the exterior of the tumour The tumour is pale in colour with the cross section showing whiter material at the core of the tumour
	67	Liver, kidney and tumors harvested but weights not recorded (NR) in error	26/04/2009	32	Ulcerated tumour	10						48.6	177.4	10.2	5.36	21.3	NR	NR	NR	1.5	Approximately 40% of the tumour exterior is covered by skin. Scab damage is visible on the uppermost surface, with virtually no penetration into the interior Possible evidence of blood vessels on the exterior of the tumour. The tumour is pale in colour and the cross section shows a white core at the centre.

APPENDIX 4: Individual PFBA Mouse Data Points

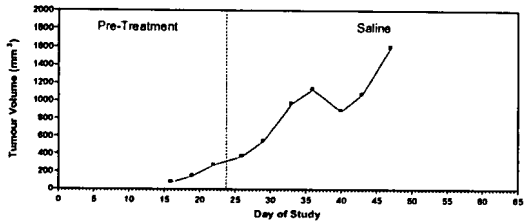
HT-29 Tumours							Tissue Concentrations of PFBA (µM)					Clinical Chemistry Parameters				Terminal Measurements (g)			Tumour Details (NBF-Fixed)		
Treatment	Mouse ID	Study Diary Comments/SSO and SO Comments	Date Mouse Terminated	Day of study	Reason for Termination of mouse	Number of Doses of PFBA	Blood Harvest 1: Day 22: (following 5 doses at 350mg/kg)	Blood harvest 2: Day 43: (5 doses at 350mg/kg + 20 doses at 250mg/kg)	Blood harvest 3: Day 57 (5 doses at 350mg/kg + 35 doses at 250mg/kg)	Terminal Plasma: Day 64 (5 doses at 350mg/kg + 41 doses at 250mg/kg)	Tumour: Day 64 (5 doses at 350mg/kg + 41 doses at 250mg/kg)	ALT (U/L)	AST (U/L)	CRE (µmol/L)	BUN (mmol/L)	Body wt	Liver wt	Kidney wt	Tumour wt	TRS Index	Morphology
250mg/kg PFBA	68	Tumour exuded pus when measured on day 33	27/04/2009	33	Ulcerated Tumour	5 at 350mg/kg + 4 doses at 250mg/kg	105.12	ns	ns	1210.75	569.75	45.9	178.2	10.7	4.73	24.1	1.902	0.352	0.301	2.5	Possible scab on the uppermost surface of the tumour. The tumour is pale in colour and there is possible evidence of blood vessels on the exterior surface. The central core of the tumour appears to be composed of whiter material
	69		14/05/2009	50	Excessive tumour size	5 at 350mg/kg + 21 doses at 250mg/kg	ns	133.85	ns	236.64	60.69	102.1	150	8.1	6.27	25.5	2.274	0.342	1.415	1.5	Approximately 50% of the tumour exterior is covered by skin. Scab damage is visible on the uppermost surface. Possible evidence of blood vessels on the exterior of the tumour. There is a darker colour patch on the bottom surface of the tumour which projects slightly into the interior. A large percentage of the cross section shows a whiter material.
	70	Large scab on tumour, which also exceeded limit for tumour volume.	05/05/2009	41	Excessive tumour size	5 at 350mg/kg) + 12 doses at 250mg/kg	25.46	ns	ns	258.9	68.33	64.1	109	9.8	5.75	23.2	1.976	0.318	1.196	2.5	Approximately 50% of the tumour exterior is covered by skin. A large thick scab is visible on the uppermost surface, with some protrusion into the interior. The tumour is pale in colour with the cross section showing paler material than the surrounding tissue.
	73	Large hole in tumour - could not be measured accurately. Small amount of pus exuded from tumour upon dissection.	25/05/2009	61	Ulcerated tumour	5 at 350mg/kg + 32 doses at 250mg/kg	34.29	ns	132.68	164.92	43.8	149.9	109.3	8.6	7.1	23.6	2.203	0.334	0.359	2.5	Approximately 50% of the tumour exterior is covered by skin. A hole visible on the uppermost surface caused by scab damage. 60% of the cross section show paler material than the surrounding tissue
	74		28/05/2009	64	Completed study	5 at 350mg/kg + 34 doses at 250mg/kg	79.89	181.27	168.56	209.05	51.16	50.8	71.5	6.7	6.51	24.4	2.197	0.34	0.454	2.5	No Skin is visible on the surface of the tumour. There is no visible scab damage. There is possible evidence of blood vessels on the exterior surface. The tumour is pale in colour with pale white material at the centre of the tumour
	75	Stomach and SI empty of contents. Caecum contained small amount of normal content. LI empty. No build up of gas. No obvious signs of mis dose. Lower jaw of mouse canibalised by cage mates.	21/04/2009	27	Mouse found dead	5 at 350mg/kg	ns	ns	ns	ns	ns									-	Tumour not harvested
	76	Hole in tumour	27/04/2009	33	Ulcerated tumour	5 at 350mg/kg + 4 doses at 250mg/kg	113.53	ns	ns	1331.05	882.31	59.1	141.7	9	5.36	22.6	1.944	0.303	0.817	2.5	Approximately 50% of the tumour exterior is covered by skin. Scab damage is visible on the uppermost surface, with some protrusion into the interior. Possible evidence of blood vessels on the exterior of the tumour The tumour is pale in colour with the cross section showing some white material but this is not as dense as on other tumours.
	77	Large scab on tumour noted on day 40; animal carefully monitored till culled.	08/05/2009	44	Excessive tumour size	5 at 350mg/kg + 15 doses at 250mg/kg	140.86	158.15	ns	260.81	71.9	166.2	152.3	8.3	5.77	21.5	2.292	0.295	1.358	1.5	Approximately 50% of the tumour exterior is covered by skin. Considerable scab damage is visible on the uppermost surface, with protrusion into the interior. The interior of the tumour appears to be soft as there are some particles from this area floating in the NBF. The tumour is pale in colour with densely packed white core at the centre. A large scab has separated from the tumour and is floating in the pot.
	78	Tumour noted as 'squishy' on days 54, 57 and 61. Yellow pus with pungent aroma exuded from tumour when harvested.	28/05/2009	64	Completed study	5 at 350mg/kg + 34 doses at 250mg/kg	35.46	120.77	ns	178.94	73.37	30.6	85.8	13.8	5.84	25.9	2.168	0.335	0.637	4	Approximately 50% of the tumour exterior is covered by skin. The cross section shows a large hole at the centre of the tumour stretching from one side of the cross section to the other. There are some particles from the tumour floating in the NBF. Possible evidence of blood vessels on the exterior of the tumour.
	80	Scab on tumour noted on day 61	28/05/2009	64	Completed study	5 at 350mg/kg + 34 doses at 250mg/kg	39.81	55.60	120.77	80.36	49.13	48.7	59.6	7.6	6.86	27.3	2.284	0.355	0.688	1.5	Approximately 50% of the tumour exterior is covered in skin. A large scab is visible on the exterior surface but there is no sign of penetration. There is some possible evidence of blood vessels on the exterior of the tumour. The tumour is pale in colour with whiter material at the core.
	81		11/05/2009	47	Excessive tumour size	5 at 350mg/kg + 18 doses at 250mg/kg	81.29	118.67	ns	183.38	45.41	104.2	223.2	11.4	9.34	24.8	2.348	0.369	1.828	2	Approximately 50% of the tumour exterior is covered by skin. A scab is visible on the uppermost surface, with some protrusion into the interior. Possible evidence of blood vessels on the exterior of the tumour. The tumour is pale in colour with the majority of the cross section showing whiter material than the surrounding tissue. There is a small hole in the white area.
	82	Small hole evident in tumour on day 63. This was clean and dry and mouse was not distressed; animal therefore dosed on day 63 as per protocol. Animal re-assessed on day 64; hole in the tumour prevented acurate measurement. Mouse culled day 64.	30/04/2009	36	Ulcerated tumour	5 at 350mg/kg + 2 doses at 250mg/kg	15.46	ns	ns	13.15	7.90	41	132.2	9	5.78	25.2	1.564	0.32	0.294	1.5	Approximately 50% of the tumour exterior is covered by skin. A scab is visible on the uppermost surface, with some protrusion into the interior. Possible evidence of blood vessels on the exterior of the tumour. The tumour is pale in colour with the 50% of the cross section showing compressed white material.

APPENDIX 5: Individual PFHA Mouse Data Points

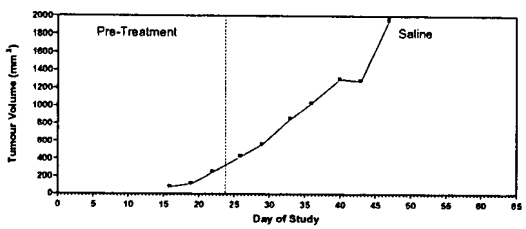
HT-29 Tumours							Tissue Concentrations of PFHA (µM)					Clinical Chemistry Parameters				Terminal Measurements (g)			Tumour Details (NBF-Fixed)		
Treatment	Mouse ID	Study Diary Comments/SSO and SO Comments	Date Mouse Terminated	Day of study	Reason for Termination of mouse	Number of Doses of PFHA	Blood Harvest 1: Day 29 (7 doses)	Blood harvest 2: Day 43 (21 Doses)	Blood harvest 3 Day 57 (35 Doses)	Terminal Plasma: (10 - 41 Doses)	Tumour: (10 - 41 Doses)	ALT (U/L)	AST (U/L)	CRE (µmol/L)	BUN (mmol/L)	Body wt	Liver wt	Kidney wt	Tumour wt	TRS	Morphology
100mg/kg PFHA	83		11/05/2009	47	Excessive tumour size	25	3.82	12.87	ns	1.44	2.37	111.2	224	27.2	9.24	23.3	1.241	0.24	1.856	2.5	Approximately 50% of the tumour exterior is covered with skin. Possible evidence of blood vessels on the exterior of the tumour. The tumour is pale in colour with a couple of darker patches which penetrate slightly into the uppermost surface. The cross section appears mostly white in colour.
	84		28/05/2009	64	Completed study	42	1.27	2.14	2.84	1.96	0.52	36.8	121.4	24.6	4.52	21.9	1.286	0.252	0.205	2	Approximately 40% of the exterior surface is covered in skin. There is slight evidence of scabbing on the exterior surface. Possible evidence of blood vessels on the exterior of the tumour. The tumour is pale in colour with white material at the core.
	85	Tumour noted as 'squishy' on day 61. Tumour exuded clear fluid when harvested.	28/05/2009	64	Completed study	42	2.94	1.46	3.58	1.15	18.32	48.8	91.8	9.2	4.67	24.7	1.364	0.322	0.788	4.5	The tumour is pale in colour with paler white patches observed both interior and exterior. There is a large hole at the centre of the tumour giving the cross section the resemblance of a conch shell. Possible evidence of blood vessels on the exterior of the tumour.
	86		28/05/2009	64	Completed study	42	3.7	10.37	ns	1.36	1.77	27.6	97	16.8	3.28	24.4	1.29	0.28	0.382	4	Approximately 50% of the tumour exterior is covered by skin. The interior of the tumour is composed of soft white material. There are fragments of the tumour from this region floating in the NBF. A slight hole is visible at the centre of the tumour. The tumour is mostly white in colour both interior and exterior.
	87	Tumour noted as 'squishy' on days 57, and 61. Pale focus in middle lobe of liver at necropsy. *Study Director's note: According to termination sheets and study diary, this mouse was terminated on 13th May, but there continue to be tumour measurements for this animal after that date.*	13/05/2009	49	Ulcerated tumour	27	2.17	4.85	17.51	260.61	29.26	46.4	99.6	39.6	7.96	25.3	1.498	0.278	1.002	2	Approximately 50% of the tumour exterior is covered by skin. A hole is visible in the uppermost surface of the tumour, possibly caused by scabbing. Possible evidence of blood vessels on the exterior of the tumour. Virtually all of the cross section is white in colour.
	88		11/05/2009	47	Excessive tumour size	25	2.93	3.26	ns	1.87	17.26	70.3	129.4	10.8	7.71	26.1	1.338	0.313	1.5	3.5	Approximately 50% of the tumour exterior is covered by skin. There is evidence of a scab on the uppermost surface of the tumour. The majority of the cross section is white in colour. A hole is visible in the centre of the tumour surrounded by soft white material. (doughnut appearance)
	89	Hole in tumour prevented accurate measurement. Mouse also appeared distressed; animal culled.	30/04/2009	36	Ulcerated tumour	14	2.62	ns	ns	0.71	0	71.3	116.2	9.2	4.89	22	1.25	0.245	0.655	2	A small patch of skin is visible on the tumour exterior. There is evidence of a scab on the exterior surface which has created a hole. Possible evidence of blood vessels on the exterior surface. The tumour is pale in colour with concentrated white material visible at the centre
	90	Liver, kidney and tumors harvested but weights not recorded (NR) in error	26/04/2009	32	Ulcerated tumour	10	2.21	ns	ns	389.84	93.81	43.7	165.6	12.4	6.08	22	NR	NR	NR	2	Approximately 60% of the tumour exterior is covered by skin. A hole is visible in the uppermost surface of the tumour, possibly caused by scabbing. Possible evidence of blood vessels on the exterior of the tumour. The tumour is pale in colour with concentrated white material visible at the centre
	91		27/04/2009	33	Ulcerated Tumour	11	4.55	ns	ns	1.83	0.24	66.1	18.2	16.6	3.04	22.4	1.239	0.266	0.9	2	No skin is visible on the exterior of the tumour. There is possible evidence of blood vessels on the exterior of the tumour. Approximately 50% of the cross section shows white material
	92		25/05/2009	61	Excessive tumour size	39	3.05	2.03	2.14	2.08	3.24	83.8	72.8	7.9	4.7	25.2	1.408	0.297	1.786	3	Approximately 50% of the tumour exterior is covered by skin. The shape of the cross section is similar to that of a rodent brain. The majority of the cross section is composed of white material in contrast to the pale exterior. A small hole is visible at the centre of the tumour.
	93	Large volume of clear pus exuded from tumour upon dissection.	25/05/2009	61	Excessive tumour size	39	7.63	1.85	3.03	2.99	13.98	98.5	84.3	7.3	5.27	24.7	1.518	0.282	2.594	4	Approximately 50% of the tumour exterior is covered by skin. The core of the tumour is composed of concentrated white matter with a large hole at the centre. (doughnut appearance)
	94	Tumour was too small to measure on day 31, despite being measurable on five previous occasions. Tumour was again measurable on all other days, but accuracy of measurements on days 36 and 40 questioned as tumour was small.	28/05/2009	64	Completed study	42	9.8	3.74	4.03	1	2.76	31.2	87.6	11	4.84	23.7	1.227	0.311	0.402	2	Possible evidence of blood vessels on the exterior surface. The tumour is pale in colour with a concentrated core of white material visible in the cross section.

APPENDIX 6: Individual Control Tumour Volume Graphs

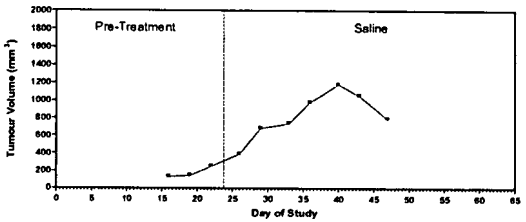
CXR0788: Mouse 53



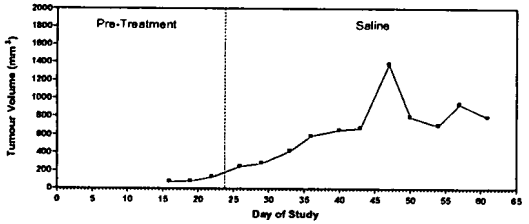
CXR0788: Mouse 54



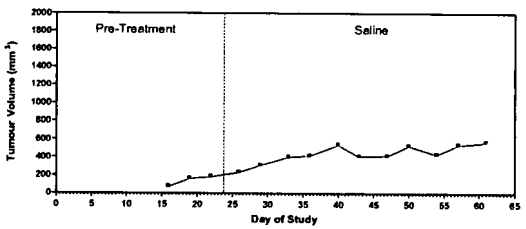
CXR0788: Mouse 56



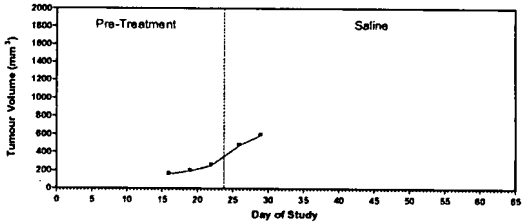
CXR0788: Mouse 57



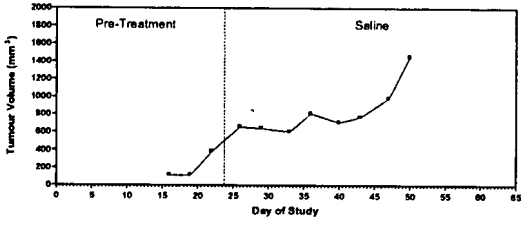
CXR0788: Mouse 58



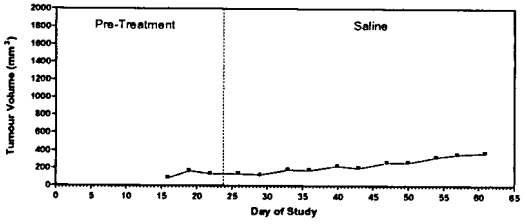
CXR0788: Mouse 60



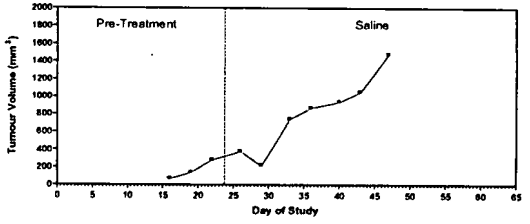
CXR0788: Mouse 61

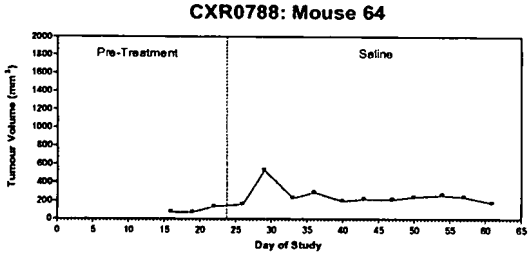


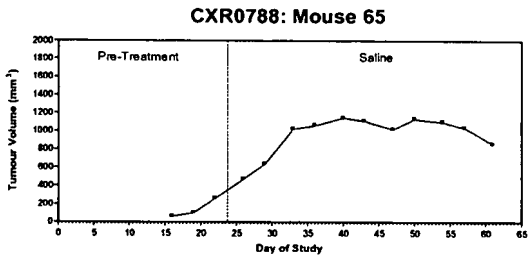
CXR0788: Mouse 62

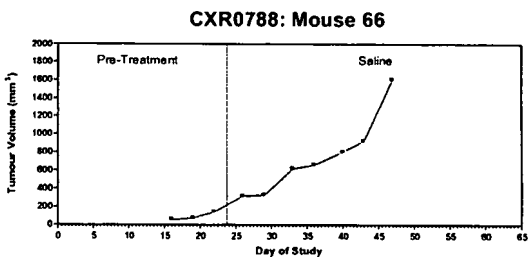


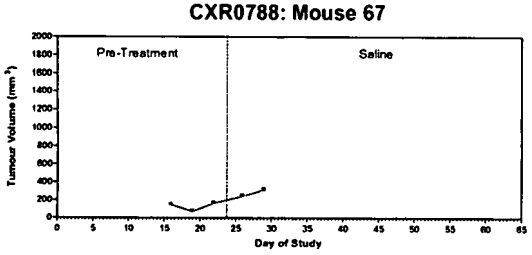
CXR0788: Mouse 63



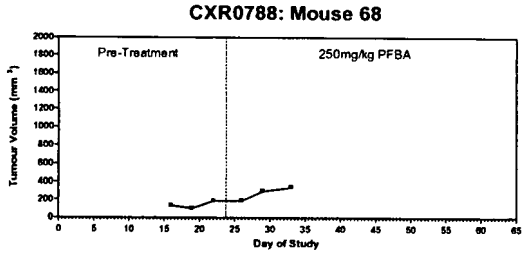

CXR0788: Mouse 64
Pre-Treatment
Saline
Tumour Volume (mm³)
Day of Study

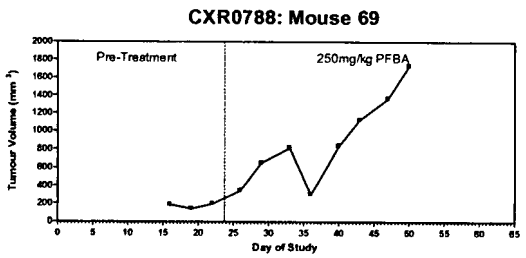

CXR0788: Mouse 65
Pre-Treatment
Saline
Tumour Volume (mm³)
Day of Study

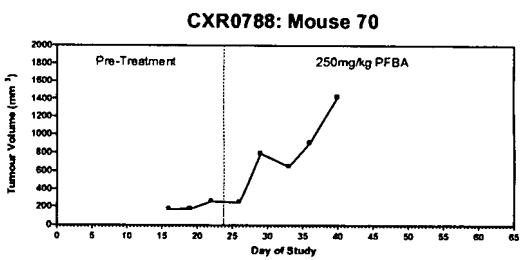

CXR0788: Mouse 66
Pre-Treatment
Saline
Tumour Volume (mm³)
Day of Study

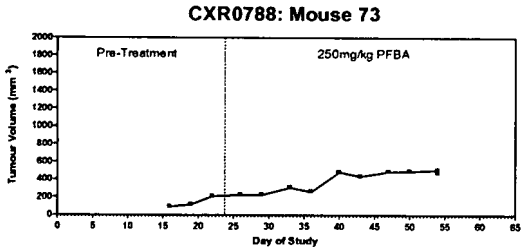

CXR0788: Mouse 67
Pre-Treatment
Saline
Tumour Volume (mm³)
Day of Study

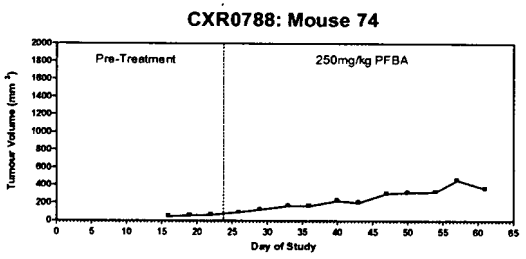
APPENDIX 7: Individual PFBA Tumour Volume Graphs

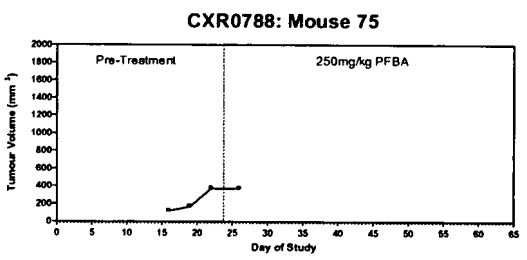


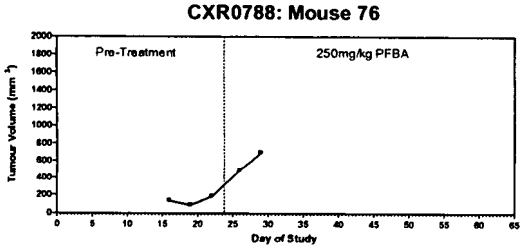


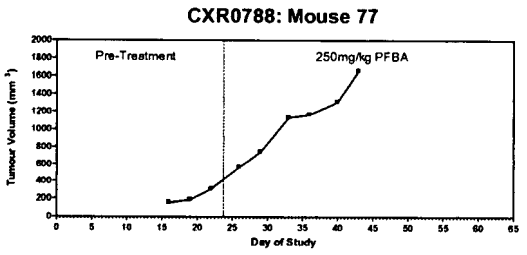


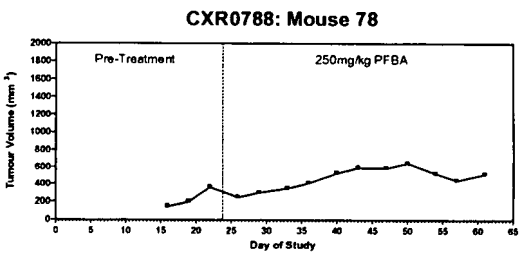


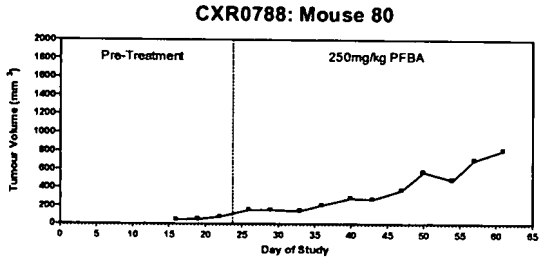


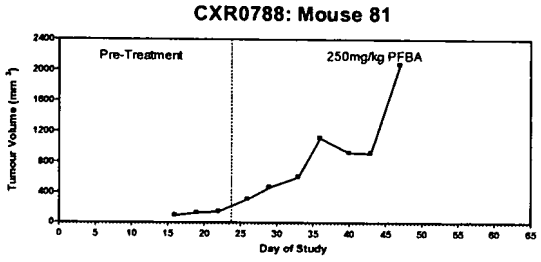


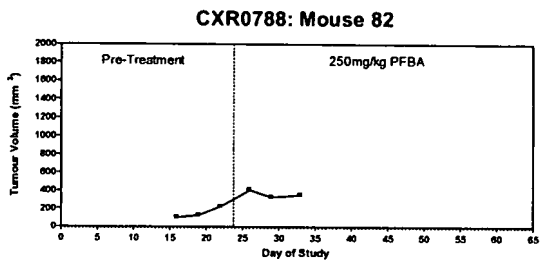




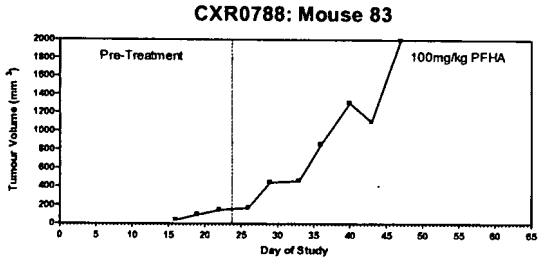


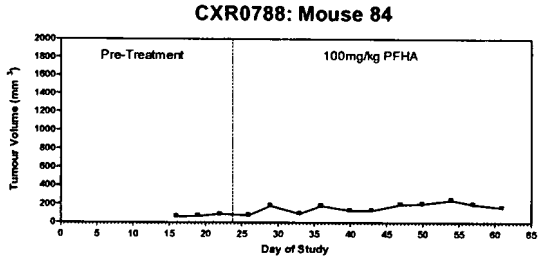

CXR0788: Mouse 80
Pre-Treatment
250mg/kg PFBA
Tumour Volume (mm 3)
Day of Study

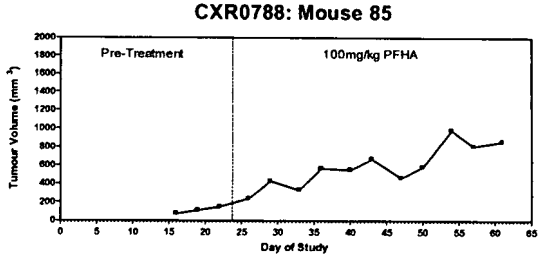

CXR0788: Mouse 81
Pre-Treatment
250mg/kg PFBA
Tumour Volume (mm 3)
Day of Study

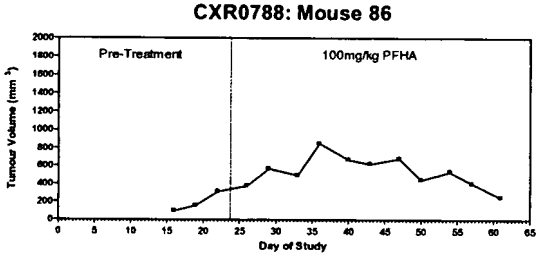

CXR0788: Mouse 82
Pre-Treatment
250mg/kg PFBA
Tumour Volume (mm 3)
Day of Study

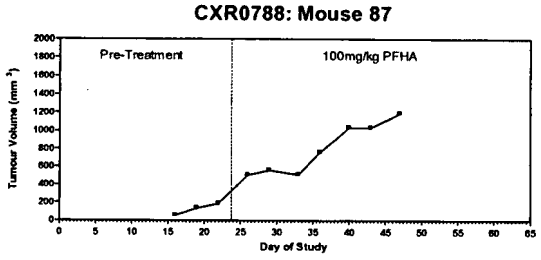
APPENDIX 8: Individual PFHA Tumour Volume Graphs

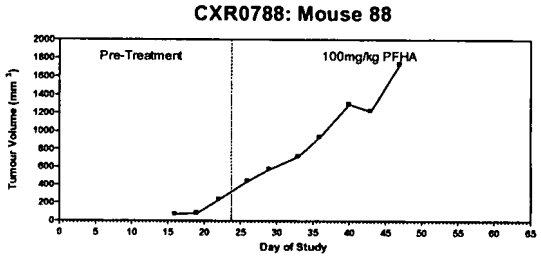


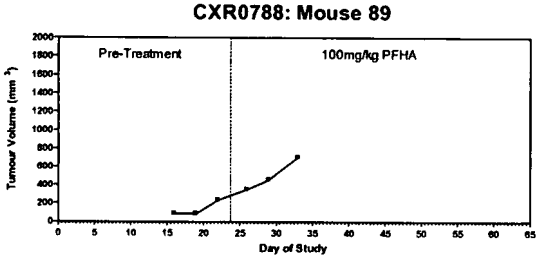


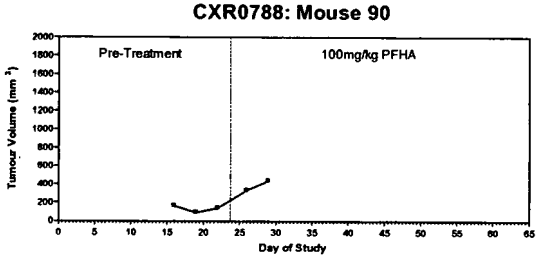


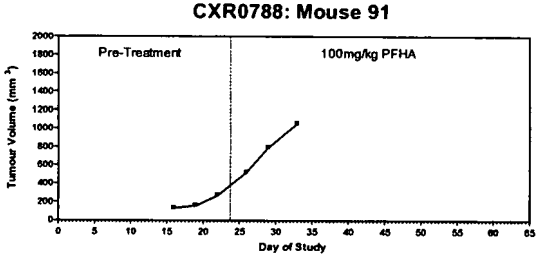


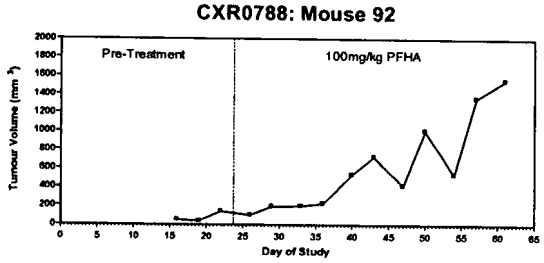


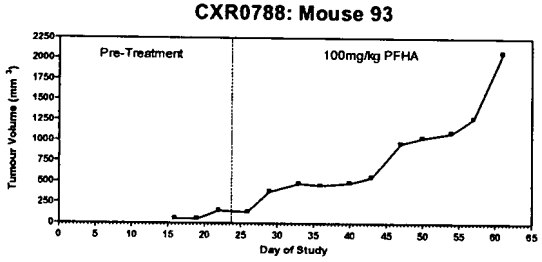


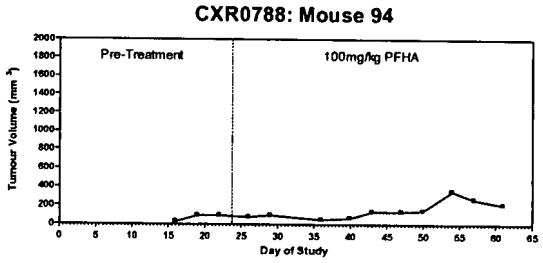







CXR0788: Mouse 92
Pre-Treatment
100mg/kg PFHA
Tumour Volume (mm 3)
Day of Study


CXR0788: Mouse 93
Pre-Treatment
100mg/kg PFHA
Tumour Volume (mm 3)
Day of Study


CXR0788: Mouse 94
Pre-Treatment
100mg/kg PFHA
Tumour Volume (mm 3)
Day of Study